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Colonization history of Majorca Island by the European rabbit, *Oryctolagus cuniculus*, and the Iberian hare, *Lepus granatensis* (Lagomorpha: Leporidae)

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Short title: Colonization of Majorca by rabbits and hares

Abstract

The Mediterranean islands have a long history of human-mediated introductions that resulted in frequent replacements of their fauna and flora. While these histories are sometimes well documented or may be inferred by paleontological studies, the use of phylogenetic and population genetic reconstruction methods provide a complementary perspective for answering questions related to the history of insular species. In this study, we infer the colonization history of Majorca (Balearic Islands) by the European rabbit (*Oryctolagus cuniculus*) and the Iberian hare (*Lepus granatensis*) using sequence variation of the mitochondrial DNA Control Region from continental and insular specimens (total of 489 sequences). Additionally, the taxonomic identity of Majorcan *L. granatensis* was confirmed using a diagnostic nuclear marker. For both Majorcan rabbits and hares, genetic diversity was comparable to the continental populations, suggesting introduction of multiple lineages. Two Majorcan haplogroups were found in hares, which likely correspond to two introduction events. Rabbits from Majorca were identified as belonging to the subspecies *O. c. cuniculus*, and may have been originated both from Iberian and French populations. The molecular estimates of the timing of the colonization events of the Majorcan lagomorphs are consistent with human-mediated introductions by early settlers on the islands.

Keywords: Control Region; Phylogeny; Island phylogeography; Introductions; Mediterranean islands; Iberian Peninsula; Lagomorphs.

Introduction

Islands are considered natural laboratories for the study of evolution, particularly of colonizations and extinctions (Lomolino, 2000). Understanding the framework of this process and distinguishing natural from human-mediated introductions are often challenging because of the lack of historical records of introductions. The advent of molecular genetics has given invaluable contributions to better understand the native or alien nature of fauna and flora populating islands, and to clarify the impact of the founder event in the genetic diversity of these taxa (e.g. Sly, Townsend, Rimmer, Townsend, Latta & Lovette, 2011).

The Balearic Islands are an archipelago located in front of the eastern coast of the Iberian Peninsula. Due to their geographical position in the Western Mediterranean Sea, they were of great importance for ancient navigation for at least four thousand years (see e.g. Bellard, 1995). These islands have been settled throughout their history by various civilizations, such as Punics and Romans (e.g. Alcover, 2008), which have left important marks in the biotic and abiotic features of the territory (e.g. Uerpmann, 1971; Alcover, 1982). Contradicting previous suggestions that the colonization of the Balearics by extant mammals was very old, Alcover (1982) proposed that most of the fauna presently occurring in the archipelago was introduced into the islands only recently by man. This idea is supported by the absence of paleontological findings representative of any presently living mammal of the Balearics older than the Holocene (Adrover, 1966). According to Alcover (1982) and Bover, Quintana & Alcover (2008), apart from bats and birds, only three terrestrial mammals (the Balearic Cave Goat, *Myotragus balearicus*, Bate 1909, the Majorcan giant dormouse, *Hypnomys morphaeus*, Bate 1918, and the Balearic shrew, *Nesiotites hidalgo* Bate 1944), a lizard (*Podarcis lilfordi* Günther 1874) and one amphibian (*Alytes muletensis* Sanchíz and Adrover 1977), existed in

Majorca until the Holocene. Only with the arrival of humans did other non-flying mammals colonize the island.

The first known written record of the presence of rabbits in the Balearic Islands reports a rabbit plague in the Majorca and Menorca islands by the time of the emperor Augustus Caeser (*ca.* 2000 BP), which can be found in Pliny's the Elder book, "Naturalis Historia". Pliny based his writings on other books dating from that time and on stories told by others. Thus, the information in his book may not be reliable. Rabbits also have mythological importance in Majorca and legends of the existence of a rabbit-hare like species, locally named 'esquirol', persist until today. However, nothing can be interpreted from these writings regarding the introduction of lagomorphs (hares and rabbits) into the island, which could date back to the arrival of the Romans (who conquered the island at ca. 2100 BP) or to the time of the Talayotic culture (settlers that lived on the island before the Romans). Reumer and Sanders (1984), based on palaentological data, suggested that rabbits were present in Menorca since 3350-3250 BP, but this date is tentative, since no radiocarbon dating was performed. Regardless of the age of the first introductions of lagomorphs into Majorca, anecdotal evidence suggests that more recent introductions for hunting purposes are known to have occurred, and there is an original breed of rabbits recognized in the Balearic islands (named "Conill pagès d'Eivissa").

Based on morphological data, Palacios and Fernández (1992) described Majorcan hares as a subspecies of the Iberian hare (*Lepus granatensis* Rosenhauer 1856), *L. g. solisi*. In the mainland this species has a broad distribution across the Iberian Peninsula except in the northeastern region where it is replaced by *L. europaeus* Pallas 1778 and in the Cantabrian mountain chain where *L. castroviejoi* Palacios 1977 is found instead (see Alves, Ferrand, Suchentrunk & Harris, 2003 and references therein). Rabbits are classified in genus *Oryctolagus* which includes a single species

(*Oryctolagus cuniculus* Linnaeus 1758) that originated in the Iberian Peninsula and naturally colonized Central Europe. Two subspecies are recognized, *O. c. algirus* Loche 1858, distributed in the south-western part of Iberia, and *O. c. cuniculus* Linnaeus 1758, present in the northeast of Iberia, Europe, and in several other locations around the world where it has been introduced by man, such as Australia and South America (see e.g. Branco, Ferrand & Monnerot, 2000, references therein; Ferrand, 2008). *O. c. algirus* is the rabbit subspecies that is thought to inhabit the Balearic Islands (Gibb, 1990).

In this study, the history of colonization of Majorca by hares and rabbits and their likely taxonomic status were inferred using molecular data. The results were compared to historical documentation and previous studies. We aim at contributing to a better understanding of the history of colonization of Majorca by tracing the origins of Majorcan hares and rabbits and disentangling hypotheses of human-mediated or natural colonizations.

Material and Methods

Sampling and laboratory procedures

Tissue samples of 25 wild rabbits and 25 hares, mostly from road kills, were collected by Autonomic Environment agents around the Majorcan localities of Llucmajor and Porreres (Fig. 1). DNA from ear and muscle tissue was extracted using Genomic DNA Tissue Kit (EasySpin) following manufacturer's instructions. A fragment of the mitochondrial DNA (mtDNA) Control Region was PCR amplified using primers LCRSEQ (5'-CACCATCAGCACCCAAAG-3') (Melo-Ferreira, Boursot, Randi, Kryukov, Ferrand & Alves, 2007) and LEPD2H (5'-

ATTTAAGAGGAACGTGTGGG-3') (Pierpaoli, Riga, Trocchi & Randi, 1999), both for rabbits and hares. In addition, DNA was extracted from 42 other wild rabbits collected from several locations in the Iberian Peninsula (Fig. 1; Supporting Table 1). A similar mtDNA Control Region fragment was amplified in these samples using primers Pro1 (5'-CCACCATCAGCACCCAAAGCT-3') and NC4 (5'-ATGGCCCTGAGGTAAGAACC-3') (Mougel, 1997; Branco, Monnerot, Ferrand & Templeton, 2002). Cleaning of PCR products was performed with ExoSap-IT (according to USB protocol) and then purified using Sephadex™ G-50 Medium (GE Healthcare) in CENTRI-SEP™ columns, prior to sequencing. Sequencing was performed using the BigDye Terminator cycle sequencing protocol and using the LCRSEQ and NC5 (5'-CTTTAATAAAACTCAAGTACTTC-3') (Mougel, 1997; Branco et al., 2002) primers.

Due to the documented mtDNA introgression in hares (Alves et al., 2003; Melo-Ferreira, Boursot, Suchentrunk, Ferrand & Alves, 2005; Melo-Ferreira et al., 2007; Alves, Melo-Ferreira, Freitas & Boursot, 2008), and the subsequent inaccuracy of using mtDNA alone for species identification (Alves, Harris, Melo-Ferreira, Branco, Ferrand, Suchentrunk & Boursot, 2006), a diagnostic nuclear marker (UCP2, Uncoupling Protein 2) was amplified in all hare samples using primers LUCP2e6F1 (5'-TGCCGAGCTGGTCACCTAT-3') and LUCP2i67R1 (5'-GACTTCCACTGAGGCCCTAAAA-3') (Melo-Ferreira, Alves, Freitas, Ferrand & Boursot, 2009).

Data analysis

The sequence dataset of both rabbits and hares was complemented with the inclusion of 28 rabbit and 429 hare sequences from the mainland recovered from GenBank (see Supporting Tables 1a and 1b for GenBank Accession Numbers of Iberian hare and European rabbit sequences). The sequences of mtDNA Control Region of Majorcan hares and rabbits were first aligned against a

dataset of sequences known to be representatives of the major lineages of these species in the mainland (for hares, 10 representatives of the native and 10 of the introgressed lineage were used and for rabbits 10 representatives of each subspecies were included; see Suporting Table 1), using ClustalW multiple alignment implemented in BioEdit v7.0.5.3 (Hall, 1999). The phylogenetic reconstruction was performed in MEGA5 (Tamura, Peterson, Peterson, Stecher, Nei & Kumar, 2011), using the maximum-likelihood (ML) and Neighbour-Joining (NJ) methods under the GTR and HKY mutation models with gamma distributed site heterogeneity rate (applied to hares and rabbits datasets, respectively) as determined in jModelTest0.1 (Guindon & Gascuel, 2003; Posada, 2008). Branch support was assessed using 500 bootstrap replicates. In the case of hares, this allowed determining whether the mtDNA lineage of the Majorcan specimens was the native lineage or that introgressed from *L. timidus*, and in the latter case, to which sub-lineage it belonged (Melo-Ferreira et al., 2007). For rabbits, the phylogenetic reconstruction allowed assessing if Majorca rabbits belonged to mtDNA clade A (*O. c. algirus*) or clade B (*O. c. cuniculus*) (Branco et al., 2002). The relationships among Majorcan and continental haplotypes was also examined using median-joining (MJ) networks (Bandelt, Forster & Röhl, 1999) with MP optimization (Polzin & Daneschmand, 2003), produced in NETWORK v4.5.1.6 (available at http://www.fluxus-engineering.com) in an extended dataset combining the mtDNA-CR sequences produced in this study and previously published ones - 429 *L. granatensis* specimens from the Iberian Peninsula (Melo-Ferreira et al., 2007; Melo-Ferreira, Alves, Rocha, Ferrand & Boursot, 2011), and 28 *Oryctolagus cuniculus* from France (see GenBank Acc. Nrs. in Supporting Table 1) for a total of 60 rabbit sequences from the European mainland.

Nucleotide diversity (π; Nei, 1987), the proportion of segregating sites in a sample (θ_S; Watterson, 1975) and haplotype diversity (*h*) were estimated in ARLEQUIN v3.11 (Excoffier, Laval & Schneider, 2005) using the extended dataset. The mismatch distributions were calculated using the

same software, and fitted to the Sudden Expansion Model (Rogers & Harpending, 1992). This distribution is usually multimodal in populations at equilibrium, as it represents the stochastic shape of gene trees, but generally unimodal in situations of recent demographic expansion (Slatkin & Hudson, 1991; Rogers & Harpending, 1992) or range expansions with high levels of migration between neighboring demes (Ray, Currat & Excoffier, 2003; Excoffier, 2004). The frequency spectrum of mutations was examined using Tajima's D (Tajima, 1989) and Fu's Fs (Fu, 1997).

The time-frame of colonization of Majorca by rabbits and hares was estimated using i) the time of the most recent common ancestor (TMRCA) of the Majorcan clades, and ii) the TMRCA of the clades conveying Majorcan and the most closely related continental haplotypes. Estimates of the TMRCA were performed using BEAST v1.7.4 (Drummond, Suchard, Xie & Rambaut, 2012) on three separate datasets corresponding to the major lineages detected (one for rabbits and two for hares). The best-fitted mutation model was determined by jModelTest v0.1.1 (Guindon & Gascuel, 2003; Posada, 2008) using the corrected Akaike Information Criterion (AICc). This best model or, if not implemented in BEAST, the next-most complex model available, and a strict molecular clock was assigned to the data. The demographic model selected was the Bayesian skyline plot (BSP), which imposes fewer demographic prior assumptions than the remainder of the available models (Drummond, Rambaut, Shapiro & Pybus, 2005). Several runs with different chain lengths were performed in order to assess the consistency of the estimates across independent runs. The final analyses were run three times with a number of iterations that varied between 50 and 150 million depending on the run length needed to obtain stability of the Markov Chain and high effective sample sizes of the parameter estimates. TRACER v1.5 (Rambaut & Drummond, 2007) was used to assess convergence of the runs. The first 10% of the samples of the Markov Chain of each run were discarded as burn-in and the tree files of the independent runs were combined using LogCombiner v1.7.4, included in the BEAST package. The resulting trees were then visualized in FigTree v1.3.1.

Melo-Ferreira et al. (2007) estimated a divergence rate of 0.158 per million years (Myr) for the concatenated fragments of cytochrome b and D-loop, from which a divergence rate of 0.312 per Myr for the D-loop can be derived. This rate was also used for the rabbit analysis.

The demographic history of the Majorcan lineages was further explored using the Bayesian Skyline plot and four additional demographic models were implemented in BEAST v.1.7.4: constant population size, exponential growth, logistic growth and expansion growth. The fit of the different models was compared by calculating the Bayes Factor (BF) (Newton & Raftery, 1994) from the ratio of the marginal likelihoods of the different models given by the harmonic mean estimator (Suchard, Weiss & Sinsheimer, 2001) as implemented in TRACER v1.5. Since the harmonic mean estimator may perform poorly, the path sampling method was in addition used to calculate marginal likelihoods, and the Bayes Factors of the different models was again estimated (Baele, Lemey, Bedford, Rambaut, Suchard & Alekseyenko 2012).

Results

Genetic diversity and relationship between continental and Majorcan haplotypes

All analysed Majorcan hare samples were assigned as *L. granatensis* according to the diagnostic nuclear marker (UCP2 locus) - a 23 bp deletion was present in both alleles of this locus in all analysed individuals (Melo-Ferreira et al., 2009). The phylogenetic reconstruction based on control region sequences (450 bp) showed that 52% of analysed *L. granatensis* from Majorca had native mtDNA haplotypes and 48% possessed the *timidus*-like mtDNA, similar to the observed frequency in northern Iberia (Melo-Ferreira et al., 2005) (Fig. 2a). A total of eight different haplotypes were

identified among the 25 hare samples from Majorca (five of *granatensis*-type and three of *timidus*-type), and a high nucleotide diversity was found ($\pi_{(\%)}$ = 6.56; see Table 1), which reflected the divergence between native and introgressed haplotypes. Combining these sequences and those from various Iberian populations (Fig. 3; Supporting Fig. 1), the continental haplotypes that most resembled haplotypes of the Majorcan native *L. granatensis* were those from the population of Caceres (Fig. 3a; Supporting Fig. 1a). For *L. granatensis* with *timidus*-like mtDNA, the closest continental haplotype to the Majorcan haplotypes was detected in Zaragoza (Fig. 3b). Analysing the two evolutionary lineages separately, diversity among Majorcan haplotypes (native: h = 0.410, $\pi_{(\%)}$ = 0.16, $\theta_{S(\%)}$ = 0.21; *timidus*-like: h = 0.758, $\pi_{(\%)}$ = 0.63, $\theta_{S(\%)}$ = 0.67) was generally lower than that observed in the continent (h = 0.987, $\pi_{(\%)}$ = 2.44, $\theta_{S(\%)}$ = 3.62; h = 0.972, $\pi_{(\%)}$ = 3.83, $\theta_{S(\%)}$ = 3.02; for native and *timidus*-like *L. granatensis* respectively) (Table 1).

The ML and NJ phylogenetic reconstruction suggested that all Majorcan rabbits belonged to mtDNA clade B, which has been attributed to the subspecies *O. c. cuniculus*, native to north-eastern Iberia and France (Branco et al., 2002) (Fig. 2b). Therefore, in the subsequent analyses, a dataset with only clade B individuals from the continental range of the species was added. Nine haplotypes were found among the analysed Majorcan rabbits and levels of sequence diversity were comparable to those found in the native range of the subspecies *O. c. cuniculus* (Table 1). When comparing the haplotypes found in Majorca with those from the Iberian Peninsula and French populations (Fig. 4; Supporting Fig. 2), the Majorcan rabbits do not form a monophyletic group. Two similar haplotypes (hereafter named I) were more closely related to French haplotypes, and two individuals (II) to a haplotype that included individuals from Zaragoza, Benavente and France. Majorcan individuals shared the same haplotype with specimens from the continent twice. In one case, a haplotype (III) harboured one Majorcan individual and one French individual. In the other case, a haplotype (IV)

included four Majorcan individuals and one individual from Lleida. Finally, one last haplogroup (V) was highly divergent from the other sampled rabbit haplotypes.

Demographic analyses and Time of the Most Recent Common Ancestor

The mismatch analysis of both Majorcan native and *timidus*-like *L. granatensis* did not reject the sudden expansion model (Supporting Fig. 3). However, although Tajima's D value was negative in these two groups (Table 1) both values were not significant ($P > 0.05$). On the other hand, Fu's F_S values were positive, both for native and *timidus*-like hares, but again neither was significant ($P > 0.02$) (Table 1). The Bayesian Skyline analysis of *timidus*-like *L. granatensis* did not suggest recent expansion (Fig. 5) and, in the case of native *L. granatensis*, the MCMC failed to converge after multiple runs (e.g. 200 million generations), likely due to the low levels of variation present in the alignment (this plot is not shown). The Bayes Factor comparison of the different demographic models did not strongly favour any of the demographic expansion models over the constant population size model for both the Majorcan *timidus*-like and native *L. granatensis* datasets (largest 2ln(BF) favouring a growth model ~ 2.5; see Kass & Raftery, 1995 for a description of Bayes factors) – Supporting Tables 2 and 3.

The mismatch analysis of the sequences of Majorcan rabbit specimens showed a multimodal distribution, rejecting the sudden expansion model (Supporting Fig. 3). Tajima's D and Fu's F_S values were both positive but neither was significant ($P > 0.05$ and $P > 0.02$, respectively) (Table 1). The Bayesian Skyline Analysis did not suggest recent population expansion (Fig. 5) and, again, no strong support was obtained for the expansion models relative to the constant population size model (largest 2ln(BF) favouring a growth model ~ 2.2; see Kass & Raftery, 1995 for a description of Bayes factors) – Supporting Tables 2 and 3.

Estimates of the dates of introduction of hares and rabbits in Majorca were derived from the time of the most recent common ancestor (TMRCA) both for the Majorcan clades and for the closest continental haplotype (Figs. 3 and 4). For native *L. granatensis* haplotypes the HPD 95% confidence interval of the TMRCA of the Majorcan variants goes from 27500 years BP to 4000 years BP, while the TMRCA with the closest continental haplotype is between 71000 BP and 28000 BP (Fig. 3a). In the case of *timidus*-like *L. granatensis* the HPD 95% values of the Majorcan TMRCA, range from 49000 BP to 15500 BP, while that including the continental haplotypes is 72500 to 23000 years BP (Fig. 3b).

The trees obtained from the analysis using BEAST suggests that at least five independent rabbit introductions may have occurred (Fig. 4), in accordance with the Network analyses (Supporting Fig. 3). Whenever the Majorcan haplotypes did not form a monophyletic group (haplogroups I and IV) or the colonization event was represented by a single individual (haplogorup III) the TMRCA was only calculated including the more closely related continental specimen. Overall, the 95% HPD intervals varied between 170000 years BP (group V) and the present (groups III). The detailed time estimates for the colonization of all rabbit haplogroups and the 95% HPD are shown in Fig. 4.

Discussion

The nature of Majorcan hares and rabbits

In accordance with previous descriptions of Majorcan hares (Palacios & Fernández, 1992), our results on the analyzed nuclear gene suggest that the hares included in this study are all Iberian

hares, *L. granatensis*. In addition, based on mtDNA analyses, the genetic variation detected among Majorcan hares is well within and not divergent from the continental populations and therefore no support was found in our data for their classification as a separate subspecies, *L. g. solisi*, as suggested by Palacios and Fernández (1992). However, since subspecific classification must rely on information from multiple sources (including morphological data), a more thorough assessment is needed to address this question.

All the Majorcan rabbits analysed here belong to the subspecies *O. c. cuniculus*, which original distribution is north-eastern Iberia and France. This contrasts with previous suggestions of introduction of the *O. c. algirus* subspecies in Majorca by the Phoenicians, which is endemic to south-western Iberia (Gibb, 1990). While it is not known whether the latter subspecies was present on the island but went extinct, our results suggest that *O. c. algirus* may be currently absent from the island, even though a more thorough sampling would be needed to confirm this hypothesis. In this work we found no evidence of the existence of the mythological "esquirol". Such belief may have been fueled by the fact that *O. c. cuniculus* is the largest of the subspecies of the European rabbit (Ferreira, 2011). Eventual sporadic island gigantism (which is unknown for Majorcan rabbits) or the presence of an extinct giant rabbit in the Balearics (classified as *Nuralagus rex*) in the fossil record (Quintana, Köhler & Moyà-Solà, 2011) may have also contributed to the myth.

Origin and time-frame of the multiple colonizations of Majorca by lagomorphs

The last time Majorca was in contact with the Iberian Peninsula, allowing for natural colonization of land mammals, was possibly during the Messinian crisis some 5-5.5 million years BP (Hsu et al., 1977; Azzaroli & Guazzone, 1980). Such natural colonization of lagomorphs seems unlikely, since previous studies suggest that rabbits and hares have been introduced after the first human arrival at

Majorca, which is thought to have occurred by the fifth millennium BP (4300-4100 BP) (see Alcover, 2008 and references therein).

The haplotype network and phylogenetic analyses, combining sequences from Majorca and continental Europe, show that neither Majorcan hares nor rabbits are monophyletic, suggesting that multiple colonizations occurred (Figs. 3 and 4; Supporting Figs. 1 and 2). A minimum of two colonizations may have occurred for hares and five for rabbits. Using the mtDNA TMRCA estimates between the Majorcan and continental haplotypes, we estimated the likely time-frame of the colonization events, and, in case of a recent colonization, determined the likely continental origin of such colonizers, bearing in mind that Quaternary glaciations induced major changes in the distribution of species (see e.g. Hewitt, 2000), including rabbits and hares (Branco et al., 2002; Melo-Ferreira et al., 2007). Hares were estimated to have colonized the island between 4000 and 72500 BP (HPD 95% CI), while overall rabbit introductions were suggested to have occurred between 170000 BP and the present (Figs. 3 and 4). Two strategies were adopted as a proxy of possible colonization times: i) the TMRCA of Majorcan clades, and ii) the TMRCA of Majorcan and closest continental relative. While ii) may tend to overestimate colonization time if we failed to sample the closest continental relative, i) may underestimate colonization time if colonizer haplotypes went extinct, or overestimate it if multiple haplotypes from the clade were originally introduced and the extant haplotypes coalesce in a continental ancestral. Our estimates must therefore be interpreted with caution, also because of the uncertainties of various sorts that derive from the use of molecular calibrations to estimate evolutionary events (Graur & Martin, 2004; Heads, 2005). This is reflected in the wide intervals of possible colonization times obtained, which may also suggest a more complex colonization history. However, these inferences provide important clues on the time-frame of colonization on a broader scale. The known first human arrival to Majorca (fifth millennium BP) falls generally within the intervals of the estimates of TMRCA of

Majorcan clades and, even if considering the TMRCA with the closest continental relative (which is possibly largely overestimated) these intervals are dramatically more recent than the last land bridge that would have allowed natural colonization 5 million years ago. Our results suggest that the extant populations of hares and rabbits in Majorca are recent and most likely result from recent human-mediated introductions.

Considering the evolutionary proximity between Majorcan and continental haplotypes, hares seem to have been introduced from two very restricted regions in the Iberian Peninsula: Caceres and Zaragoza possibly around the same time scale (95% HPD of the TMRCA estimates overlap). Majorcan rabbits seem to have been introduced into Majorca at least in five independent events possibly originating from distinct regions from Iberia and France (the regions of Benavente, Zaragoza and eventually Lleida, and France possess the most closely related haplotypes) (Fig. 4). Interestingly, the divergence of the Majorcan rabbits from the continental haplotypes is quite variable, suggesting a more recurrent dynamic of introductions during a longer period of time. This contrasts with the patterns inferred from hares, which suggest a more simple and restricted regime of introduction.

The introduction of lagomorphs in Majorca thus seems to be intimately related with human movements on the Mediterranean and settlements in Majorca in historical and more recent times. A first human settlement on Majorca likely occurred around 4300-4100 years BP (Alcover, 2008 and references therein), followed by the emergence of the Talayotic culture (end of the fourth and beginning of the third millennium BP) and then by the occupation of the island by the Romans (2073 years BP). The first settlers are thought to have introduced into Majorca four domestic species (sheep, goats, cows and pigs) and two wild rodents: the woodmouse (*Apodemus sylvaticus* Linnaeus, 1758) and the garden dormouse (*Eliomys quercinus* Linnaeus 1766) (Alcover, 2008;

Bover & Alcover, 2008). Both rodent species have been shown to have a probable Iberian or French origin (Ramalhinho & Libois 2001; Michaux, Libois, Ramalhinho & Mourois, 1998; Perez, Libois & Nieberding 2012). It is difficult to assess whether the coincidence of geographic origins of these species and hares (Iberia) and rabbits (Iberia and France) inferred in this work reflects a coincidence of introduction events. Also, it is uncertain whether rabbits were introduced during this settlement or only later by the Romans. During the Roman period, several mammals may have been introduced, as rabbits, house mouse (*Mus musculus* Linnaeus, 1758) and weasels (*Mustela nivalis* Linnaeus, 1766) (Bover & Alcover, 2008). These two civilizations may have contributed to several waves of introductions. For example, the Talayotic people are known to have acted as mercenaries in the Punic Wars, which were fought in Sardinia, Sicily, mainland Italy, the Iberian Peninsula and North Africa (2214-2096 years BP), while the expansion of the Roman empire established frequent connections to the European mainland. Thus, either by commerce or by the return of mercenaries to the island, there could be importation of animals from the continent, which may have more frequently included rabbits but also hares, according to our results. However, we cannot discard the possibility that both lagomorph species were introduced earlier, during undocumented human movements on the Mediterranean (some of our TMRCA estimates are rather old; Figs. 3 and 4), or in some cases more recently for hunting purposes (e.g. the lower bound of the 95% HPD of the TMRCA of rabbit haplogroup III and the continental haplotype is zero; Fig. 4).

Demography of Majorcan lagomorph populations

The colonization of islands usually results in a strong bottleneck of genetic diversity (due to the founder event) followed by rapid expansion, because the colonizers encounter an empty niche to inhabit and to adapt to. We tested this hypothesis for Majorcan rabbits and hares.

The genetic diversity of hares, after distinguishing the two lineages present in Majorca (native and *timidus*-like), seems to have decreased in both lineages - ($h = 0.410$, $\pi_{(\%)} = 0.16$, $\theta_{S(\%)} = 0.21$; $h = 0.758$, $\pi_{(\%)} = 0.63$, $\theta_{S(\%)} = 0.67$; native and *timidus*-like *L. granatensis*, respectively) when compared to the diversity in the continental ones ($h = 0.987$, $\pi_{(\%)} = 2.44$, $\theta_{S(\%)} = 3.62$; $h = 0.972$, $\pi_{(\%)} = 3.83$, $\theta_{S(\%)} = 3.02$; for native and *timidus*-like *L. granatensis* respectively) (Table 1), as it is expected during founder events and colonization (Nei, Maruyama & Chakraborty, 1975). We have further estimated the levels of diversity for the more closely related continental populations, using all sequences available on GenBank (10 native and 18 *timidus*-like *L. granatensis* from Caceres and Zaragoza, respectively). Again, both present higher genetic diversity than the two lineages present in Majorca (Table 1) suggesting a decrease in diversity as a result of the founder events. Following colonization, and according to the Bayes Factor comparison, the demographic model of constant population size cannot be ruled out (Supporting Tables 2 and 3). Also, although for both lineages of hares in Majorca the mismatch distribution did not reject the Sudden Expansion Model (Supporting Fig. 3), no significant deviation to mutation drift expectations were found by Tajima's D and Fu's F_S (Table 1). For rabbits, no signs of demographic expansion were found: the mismatch distribution rejected the Sudden Expansion Model (Supporting Fig. 3); Tajima's D and Fu's F_S values were both positive and not significant (Table 1) and the Bayesian Skyline Analysis did not suggest any recent population expansion (Fig. 5). In addition, demographic expansion models were not favored as attested by the Bayes Factor comparison (Supporting Tables 2 and 3). Although a single marker is being analyzed here, the lack of evidence for population expansion may also result from the fact that introductions were very recent and the genetic signals of a bottleneck are still present in the genetic diversity of Majorcan lagomorphs. However, the small number of analyzed specimens in this work may have been insufficient to properly infer the demography of the Majorcan populations

and also explain our results. Therefore, further work is needed to confirm the demographic history of both founder and post-introduction events of the Majorcan hare and rabbit populations.

Conclusion

This work shows that rabbits and hares from Majorca originated from the complex dynamics of translocations of fauna induced by the rich history of human settlements and colonizations in the Mediterranean basin. Contrarily to previous expectations, the European rabbit subspecies that inhabits the island is *O. c. cuniculus*, and we confirmed that Majorcan hares belong to the species *L. granatensis*. Given the complex dynamics of introductions found particularly for the European rabbit, this may reflect recurrent waves of faunal replacements in Majorca induced from repeated translocations. Human-mediated introductions have shaped the geographic distribution of Mediterranean fauna and future work may help understanding the detailed history and evolutionary consequences of this complex and heterogeneous process.

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References

Adrover R. 1966. Pequeño intento de lavado de las tierras de la Cueva de Son Muleta y los resultados obtenidos. *Boletín Sociedad Historica Natural Baleares* **12:** 39-46.

Alcover JA. 1982. Note on The Origin of the present mammalian fauna from the Balearic and Pityusic Islands. *Miscellania Zoologica* **6:** 141-149.

Alcover JA. 2008. The First Mallorcans: Prehistoric Colonization in the Western Mediterranean. *Journal of World Prehistory* **21:** 19-84.

Alves PC, Ferrand N, Suchentrunk F, Harris DJ. 2003. Ancient introgression of *Lepus timidus* mtDNA into *L. granatensis* and *L. europaeus* in the Iberian Peninsula. *Molecular Phylogenetics and Evolution* **27:** 70-80.

Alves PC, Harris DJ, Melo-Ferreira J, Branco M, Ferrand N, Suchentrunk F, Boursot P. 2006. Hares on thin ice: Introgression of mitochondrial DNA in hares and its implications for recent phylogenetic analyses. *Molecular Phylogenetics and Evolution* **40:** 640-641.

Alves PC, Melo-Ferreira J, Freitas H, Boursot P. 2008. The ubiquitous mountain hare mitochondria: multiple introgressive hybridization in hares, genus *Lepus*. *Philos Trans R Soc Lond B Biol Sci* **363:** 2831-2839.

Azzaroli A, Guazzone G. 1980. Terrestrial mammals and land connections in the mediterranean before and during the messinian. *Palaeogeography, Palaeoclimatology, Palaeoecology* **29:** 155-167.

Bandelt H-J, Forster P, Röhl A. 1999. Median-Joining Networks for Inferring Intraspecific Phylogenies. *Molecular Biology and Evolution* **16:** 37–48.

Baele G, Lemey P, Bedford T, Rambaut A, Suchard MA, Alekseyenko AV. 2012. Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. *Molecular Biology and Evolution* **29:** 2157-2167.

Bellard CG. 1995. The First Colonization of Ibiza and Formentera (Balearic Islands, Spain): Some More Islands out of the Stream? *World Archaeology* **26:** 442-455.

Bover P, Alcover JA. 2008. Extinction of the autochthonous small mammals of Mallorca (Gymnesic Islands, Western Mediterranean) and its ecological consequences. *Journal of Biogeography* **35:** 1112-1122.

Bover P, Quintana J, Alcover JA. 2008. Three islands, three worlds: Paleogeography and evolution of the vertebrate fauna from the Balearic Islands. *Quaternary International* **182:** 135-144.

Branco M, Ferrand N, Monnerot M. 2000. Phylogeography of the European rabbit (*Oryctolagus cuniculus*) in the Iberian Peninsula inferred from RFLP analysis of the cytochrome *b* gene. *Heredity* **85:** 307-317.

Branco M, Monnerot M, Ferrand N, Templeton AR. 2002. Postglacial dispersal of the European rabbit (*Oryctolagus cuniculus*) on the Iberian Peninsula reconstructed from nested clade and mismatch analyses of mitochondrial DNA genetic variation. *Evolution* **56:** 792-803.

Drummond AJ, Rambaut A, Shapiro B, Pybus OG. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution* **22:** 1185-1192.

Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution* **29:** 1969-1973.

Excoffier L. 2004. Patterns of DNA sequence diversity and genetic structure after a range expansion: lessons from the infinite-island model. *Molecular Ecology* **13:** 853-864.

Excoffier L, Laval G, Schneider S. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1:** 47-50.

Ferrand N. 2008. Inferring the Evolutionary History of the European Rabbit (*Oryctolagus cuniculus*) from Molecular Markers. In: Alves PC, Ferrand N and Hackländer K, eds. *Lagomorph Biology: Evolution, Ecology, and Conservation*. Netherlands: Springer.

Ferreira C. 2011. Relationships between predation risk, disease and fitness in the wild rabbit: management implications. Unpublished PhD, Universidade de Castilla la Mancha.

Fu Y-x. 1997. Statistical Tests of Neutrality of Mutations Against Population Growth, Hitchhiking and Background Selection. *Genetics* **147:** 915-925.

Gibb JA. 1990. The European Rabbit Oryctolagus cuniculus In: Chapman JA and Flux J, eds. *Rabbits, Hares and Pikas: Status Survey and Conservation Action Plan*: Gland, Switzerland: International Union for Conservation of Nature and Natural Resources. 116-120.

Graur D, Martin W. 2004. Reading the entrails of chickens: molecular timescales of evolution and the illusion of precision. *Trends in Genetics* **20:** 80-86.

Guindon S, Gascuel O. 2003. A Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Systematic Biology* **52:** 696-704.

Heads M. 2005. Dating nodes on molecular phylogenies: a critique of molecular biogeography. *Cladistics* **21** 62–78.

Hewitt G. 2000. The genetic legacy of the Quaternary ice ages. *Nature* **405:** 907-913.

Hsu KJ, Montadert L, Bernoulli D, Cita MB, Erickson A, Garrison RE, Kidd RB, Melieres F, Muller C, Wright R. 1977. History of the Mediterranean salinity crisis. *Nature* **267:** 399-403.

Kass RE, Raftery AE. 1995. Bayes Factors. *Journal of the American Statistical Association* **90:** 773-795.

Lomolino MV. 2000. A call for a new paradigm of island biogeography. *Global Ecology and Biogeography* **9:** 1-6.

Melo-Ferreira J, Alves PC, Freitas H, Ferrand N, Boursot P. 2009. The genomic legacy from the extinct *Lepus timidus* to the three hare species of Iberia: contrast between mtDNA, sex chromosomes and autosomes. *Molecular Ecology* **18:** 2643-2658.

Melo-Ferreira J, Alves PC, Rocha J, Ferrand N, Boursot P. 2011. Interspecific X-chromosome and mitochondrial DNA introgression in the Iberian hare: selection or allele surfing? *Evolution* **65:** 1956-1968.

Melo-Ferreira J, Boursot P, Randi E, Kryukov A, F. S, Nuno F, Alves PC. 2007. The rise and fall of the mountain hare (*Lepus timidus*) during Pleistocene glaciations: expansion and retreat with hybridization in the Iberian Peninsula. *Molecular Ecology* **16:** 605-618.

Melo-Ferreira J, Boursot P, Suchentrunk F, Ferrand N, Alves PC. 2005. Invasion from the cold past: extensive introgression of mountain hare (*Lepus timidus*) mitochondrial DNA into three other hare species in northern Iberia. *Molecular Ecology* **14:** 2459-2464.

Michaux J, Libois R, Ramalhinho M, Maurois C. 1998. On the mtDNA restriction patterns variation of the Iberian woodmouse (*Apodemus sylvaticus*). Comparison with other west Mediterranean populations. *Hereditas* **129:** 187-194.

Mougel F. 1997. Variation de trois types de marqueurs génétiques dans l'évolution de l'espèce *Oryctolagus cuniculus*: aspects moléculaire et relations avec la biologie et la structure des populations. Unpublished PhD PhD, Université de Paris Sud.

Nei M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press: New York.

Nei M, Maruyama T, Chakraborty R. 1975. The Bottleneck Effect and Genetic Variability in Populations. *Evolution* **29:** 1-10.

Newton MA, Raftery AE. 1994. Approximate Bayesian Inference with the weighted Likelihood Bootstrap. *Journal of the Royal Statistical Society. Series B (Methodological)* **56:** 3-48.

Palacios F, Fernández J. 1992. A new subspecies of hare from Majorca (Balearic Islands). *Mammalia* **56** 71–85.

Perez GCL, Libois R, Nieberding CM. 2013. Phylogeography of the garden dormouse *Eliomys quercinusin* the western Palearctic region. *Journal of Mammalogy* **94:** 202-217.

Pierpaoli M, Riga F, Trocchi V, Randi E. 1999. Species distinction and evolutionary relationships of the Italian hare (*Lepus corsicanus*) as described by mitochondrial DNA sequencing. *Molecular Ecology* **8:** 1805-1817.

Polzin T, Daneschmand S. 2003. On Steiner trees and minimum spanning trees in hypergraphs. *Operations Research Letters* **31** 12 - 20.

Posada D. 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25:** 1253-1256.

Quintana J, Köhler M, Moyà-Solà S. 2011. *Nuralagus rex*, gen. et sp. nov., an endemic insular giant rabbit from the Neogene of Minorca (Balearic Islands, Spain). *Journal of Vertebrate Paleontology* **31:** 231–240.

Ramalhinho M, Libois R. 2001. The karyotype of the Formentera island garden dormouse, *Eliomys quercinus ophiusae*. *Belgian Jornal of Zoology* **131** 83-85.

Rambaut A, Drummond A. 2007. Tracer v1.4. http://beast.bio.ed.ac.uk/Tracer.

Ray N, Currat M, Excoffier L. 2003. Intra-Deme Molecular Diversity in Spatially Expanding Populations. *Molecular Biology and Evolution* **20:** 76-86.

Reumer J, Sanders E. 1984. Changes in the vertebrate fauna of Menorca in prehistoric and classical times. *Zeitschrift für Säugetierkunde* **49:** 321-325.

Rogers AR, Harpending H. 1992. Population Growth Makes Waves in the Distribution of Pairwise Genetic Differences. *Molecular Biology and Evolution* **9:** 552-569.

Slatkin M, Hudson RR. 1991. Pairwise Comparisons of Mitochondrial DNA Sequences in Stable and Exponentially Growing Population. *Genetics* **129:** 555-562.

Sly ND, Townsend AK, Rimmer CC, Townsend JM, Latta SC, Lovette IJ. 2011. Ancient islands and modern invasions: disparate phylogeographic histories among Hispaniola's endemic birds. *Molecular Ecology* **20:** 5012-5024.

Suchard MA, Weiss RE, Sinsheimer JS. 2001. Bayesian Selection of Continuous-Time Markov Chain Evolutionary Models. *Molecular Biology and Evolution* **18:** 1001–1013.

Tajima F. 1983. Evolutionary relationshps of DNA sequences in finite populations. *Genetics* **105:** 437-460.

Tajima F. 1989. Statistical Method for Testing the Neutral Mutation Hypothesis by DNA Polymorphism. *Genetics* **123:** 585-595

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28:** 2731-2739.

Uerpmann H. 1971. Die Tierknochenfunde aus der Talayotsiedlung von S'Iiiot (San Lorenzo/Mallorca).

Watterson G. 1975. On the number of segregating sites in genetical models without recombination. *Theoretical Population Biology* **7:** 256-276.

Figure Legends

Fig. 1: Map of Western Europe showing the location of the island of Majorca and sampling locations in this Island and in the European mainland. Detailed information on populations' codes is provided in Supporting Tables 1a and 1b.

Fig. 2: Maximum Likelihood (ML) phylogenies showing relationships between sampled hares (a) and rabbits (b) and the known lineages that exist in each species (a subset of available continental sequences was used). ML and Neighbour Joining bootstrap values are depicted above and below branches, respectively. Majorcan individuals are in bold (detailed information of each haplotype in Supporting Table 1a and 1b).

Fig. 3: Bayesian Inference (BI) phylogenetic tree reconstructed in BEAST of the complete dataset (continental and Majorcan sequences) of the two major hare mitochondrial DNA lineages detected in Majorca: a) native *L. granatensis* b) *timidus*-like *L. granatensis*. Posterior probabilities higher than 0.5 are shown above branches. Continental clades are collapsed except those closely related to the haplotypes found in Majorca (locality is indicated). Nodes for which the times of the most recent common ancestor were inferred are depicted by hexagons: black – Majorcan clades, white: clades grouping Majorcan and the most closely related continental sequences.

Fig. 4: Bayesian Inference (BI) phylogenetic tree reconstructed in BEAST of the complete dataset (continental and Majorcan sequences) of the rabbit mitochondrial DNA lineage detected in Majorca: *O. c. cuniculus*. Posterior probability higher than 0.5 are shown above branches. Continental clades are collapsed except those closely related to the haplotypes found in Majorca (locality is indicated). Nodes for which the times of the most recent common ancestor were

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Fig. 5: Bayesian skyline plots of a) *L. granatensis* Majorcan haplotypes (including both *L. granatensis* native and *timidus*-like haplotypes), b) *L. granatensis* Majorcan *timidus*-like haplotypes, and c) *O. c. cuniculus* Majorcan haplotypes. The solid lines represent the median effective population size and dashed lines the 95% high posterior density (HPD) upper and lower bounds. In the Y-axis the effective population sizes (N_e) are scaled to the mutation rate while the X-axis represents coalescent time in years. The plot of Majorcan native *L. granatensis* haplotypes is not shown given the lack of convergence among runs.

Table 1: Estimates of sequence diversity and neutrality tests in native (nat) and introgressed (*tim*-like) hares (*L. granatensis*) and rabbits (*O. cuniculus cuniculus*).

Group	*N*	N_H	*h*	π (%)	$\theta_{(S)}$ (%)	Tajima's *D*	Fu's *Fs*
Hares							
gra, IP	429	166	0.990 (0.001)	7.26 (3.51)	4.56 (0.94)	1.37	-23.43
gra, Maj	25	8	0.793 (0.061)	6.56 (3.31)	3.19 (1.09)	3.24	14.39
nat, IP	234	104	0.987 (0.002)	2.44 (1.23)	3.62 (0.83)	-1.05	-24.03*
nat, Cac	10	8	0.933 (0.077)	2.79 (1.56)	2.58 (1.11)	-0.81	0.12
nat, Maj	13	3	0.410 (0.154)	0.16 (0.14)	0.21 (0.14)	-0.1	0.18
tim-like, IP	195	62	0.972 (0.004)	3.83 (1.89)	3.02 (0.73)	0.8	-7.74
tim-like, IP (lin.B)	95	37	0.955 (0.009)	2.15 (1.10)	2.49 (0.68)	-0.45	-7.73
tim-like, Zar (lin.B)	18	7	0.634 (0.127)	0.78 (0.47)	1.37 (0.55)	-1.69*	0.19
tim-like, Maj	12	5	0.758 (0.093)	0.63 (0.40)	0.67 (0.33)	-0.65	0.63
Rabbits							
IP	32	21	0.972 (0.014)	2.61 (1.35)	2.98 (0.98)	-0.77	-3.54
Fra	28	26	0.995 (0.011)	2.06 (1.09)	2.61 (0.89)	-0.89	-16.93
Maj	25	9	0.867 (0.037)	2.40 (1.26)	1.77 (0.64)	0.87	3.83

gra - *Lepus granatensis*; nat - *Lepus granatensis* with native mtDNA haplotypes; *tim*-like - *Lepus granatensis* with *timidus*-like haplotypes; IP – Iberian Peninsula; Fra – France; Maj – Majorca; Cac – Caceres; Zar – Zaragoza; N - number of analysed individuals; N_H - number of observed mtDNA haplotypes; *h* - haplotype diversity; π - nucleotide diversity; θ(s) - computed from the number of segregating sites. Standard deviations (SD) are shown in brackets. Significant values are indicated by an asterisk.

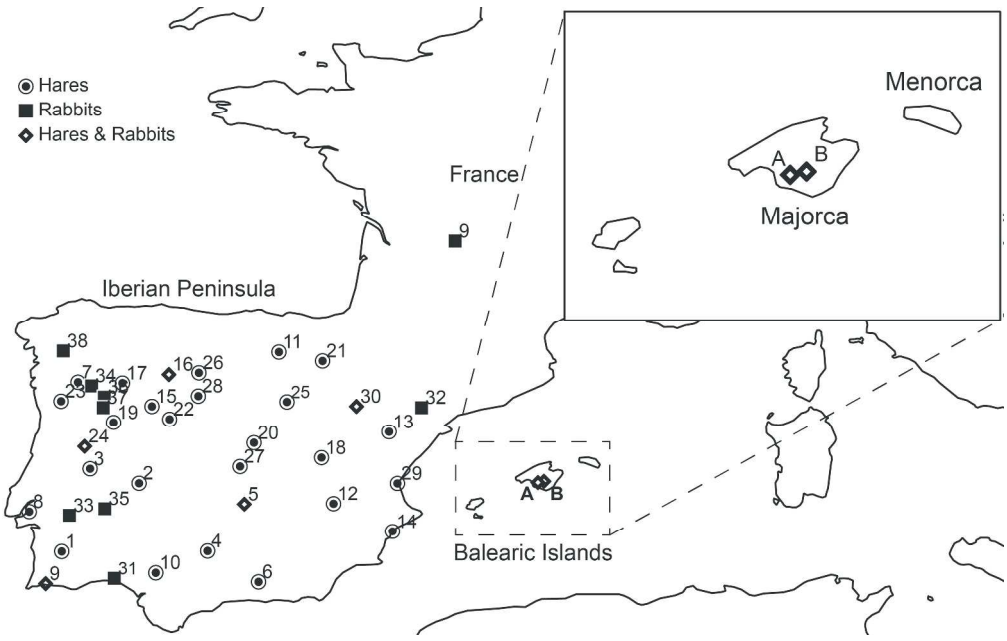
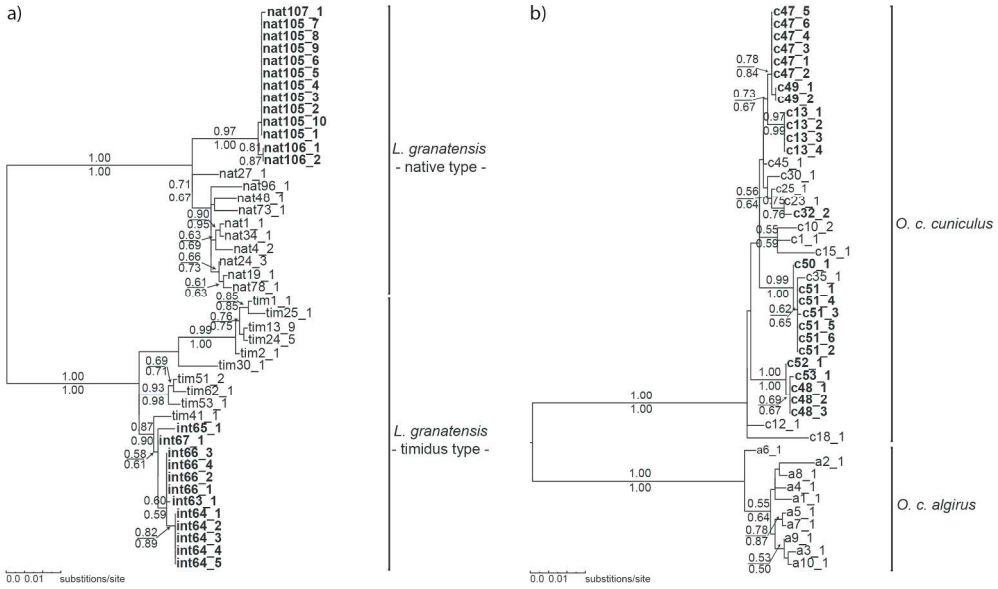


Fig. 1: Map of Western Europe showing the location of the island of Majorca and sampling locations in this Island and in the European mainland. Detailed information on populations' codes is provided in Supporting Tables 1a and 1b.
281x176mm (300 x 300 DPI)



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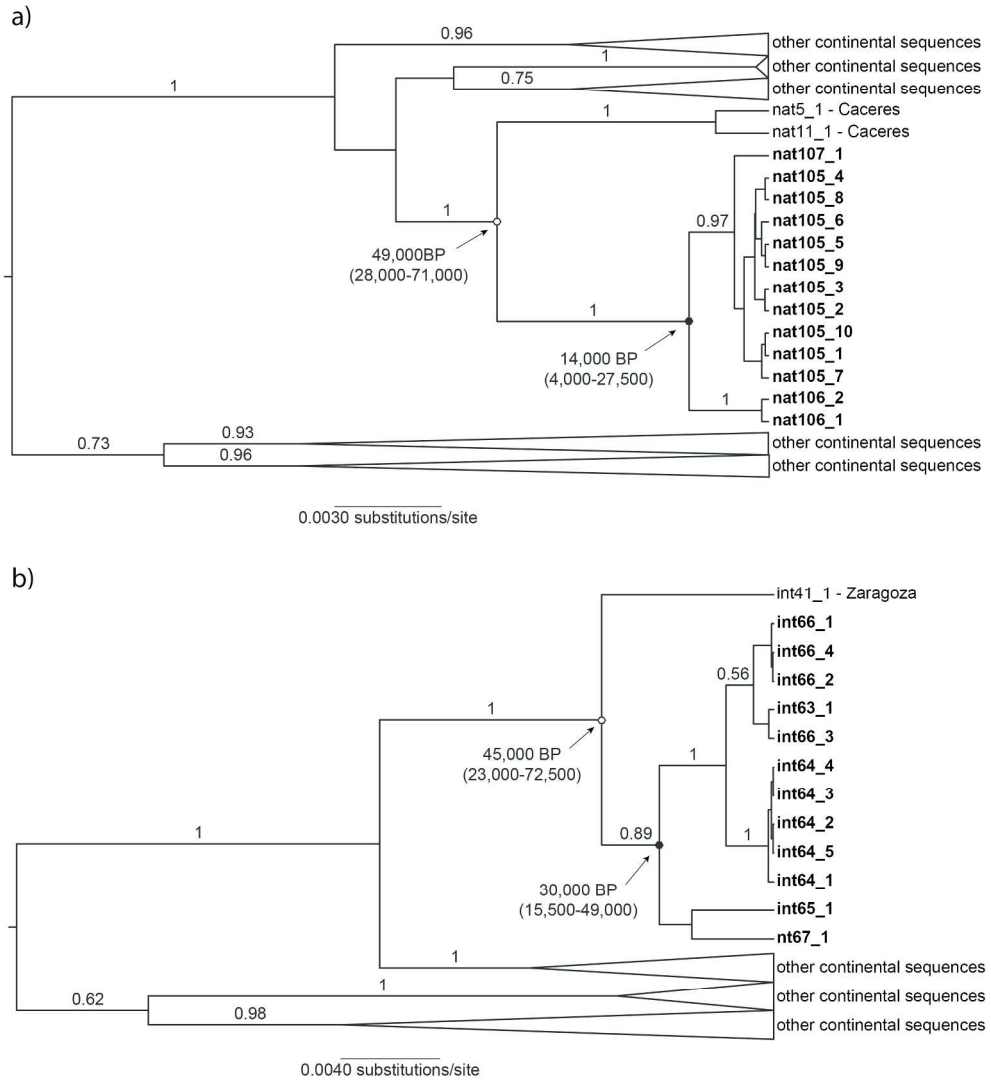
213x126mm (300 x 300 DPI)



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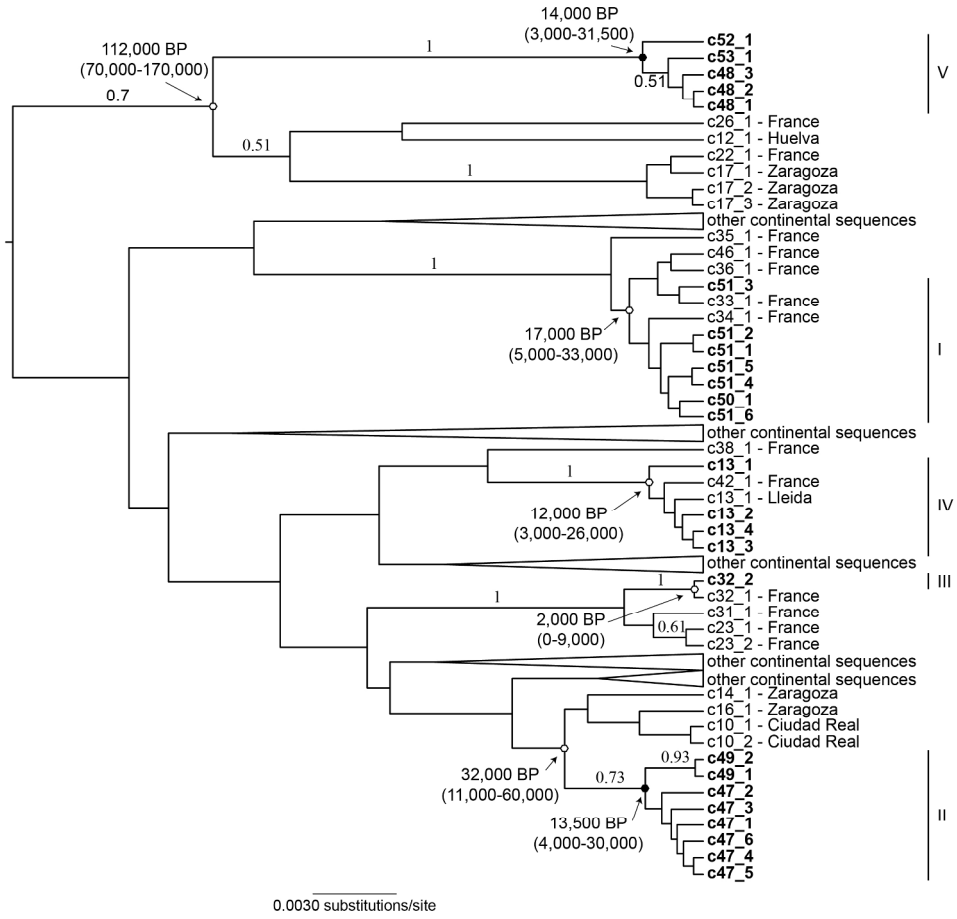
232x254mm (300 x 300 DPI)



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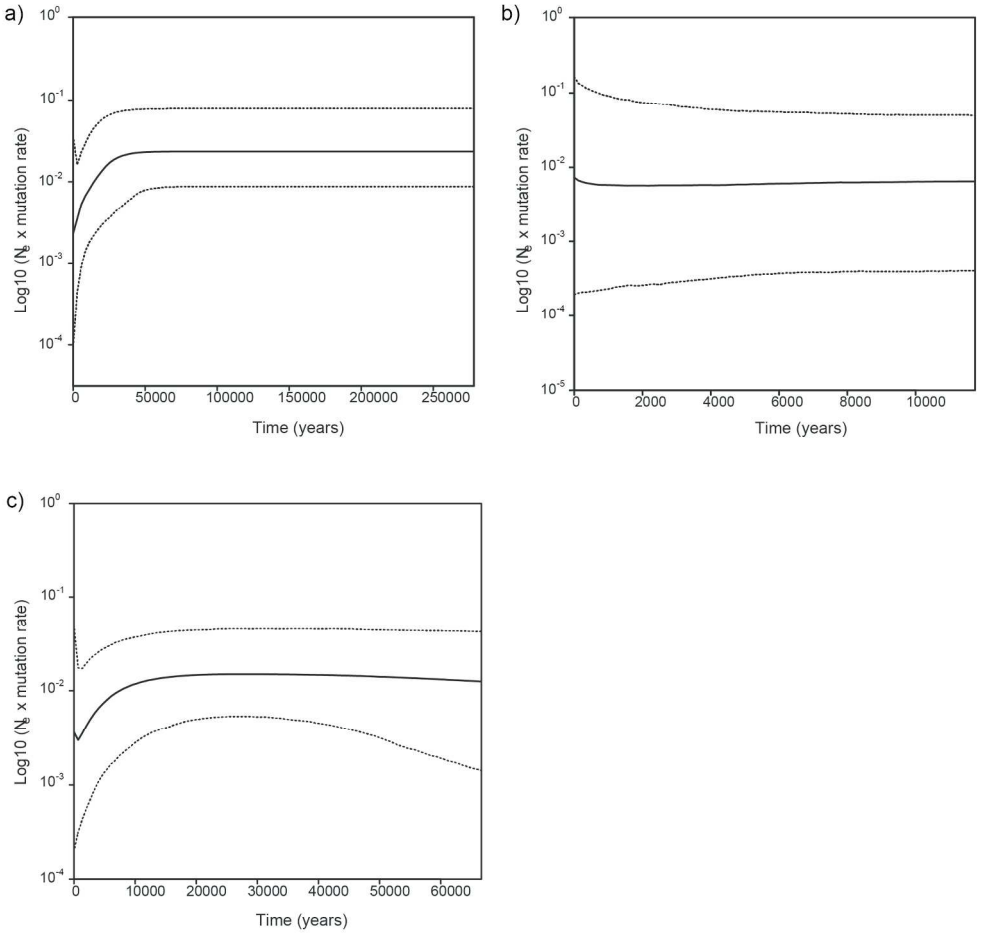
232x214mm (300 x 300 DPI)



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196x184mm (300 x 300 DPI)

SUPPORTING INFORMATION

Colonization history of Majorca Island by the European rabbit, *Oryctolagus cuniculus*, and the Iberian hare, *Lepus granatensis* (Lagomorpha: Leporidae)

Fernando A. Seixas, Javier Juste, Paula F. Campos, Miguel Carneiro, Nuno Ferrand, Paulo C. Alves, José Melo-Ferreira

Supporting Table 1a: Populations of hares sampled and respective mitochondrial DNA haplotypes (the absolute frequency is shown in parenthesis).

Locality Number	Locality	Locality Code	mtDNA haplotypes*
1	Aljustrel	Alj	nat1(5)-JF299048; nat2(2)-JF299049; nat3(3)-JF299050
2	Cáceres	Cac	nat4(1)-JF299051; nat5(1)-JF299052; nat6(3)-JF299053, JF299059; nat7(1)-JF299054; nat8(1)-JF299055; nat9(1)-JF299056; nat10(1)-JF299057; nat11(1)-JF299056
3	Castelo Branco	CBr	nat4(5)-JF299051; nat12(1)-JF299060; nat13(3)-JF299061; nat14(1)-JF299061
4	Cordoba	Crd	nat15(1)-JF299063; nat16(1)-JF299064; nat17(2)-JF299065; nat18(1)-JF299066
5	Ciudad Real	Cre	nat19(1)-JF299067; nat20(2)-JF299068,JF299072; nat21(1)-JF299069; nat22(1)-JF299070; nat23(1)-JF299071; nat24(1)-JF299073; nat25(2)-JF299074; nat26(1)-JF299075
6	Granada	Grn	nat27(5)-JF299076; nat28(4)-JF299077; nat29(1)-JF299078
7	Montalegre	Mnt	nat30(2)-JF299079; nat31(4)-JF299080; nat33(1)-JF299081; nat33(1)-JF299082
8	Pancas	Pan	nat34(6)-JF299083; nat35(2)-JF299084; nat36(1)-JF299085; nat37(1)-JF299086
9	Portimão	Ptm	nat1(4)-JF299048,JF299088; nat38(4)-JF299087; nat39(1)-JF299089; nat40(1)-JF299090
10	Sevilla	Sev	nat41(4)-JF299091, JF299094; nat42(1)-JF299092; nat43(1)-JF299093; nat44(1)-JF299095; nat45(1)-JF299096
11	Álava	Ala	nat46(9)-JF299097; nat47(1)-JF299098; int1(3)-DQ883130; int2(1)-DQ883160; int3(5)-DQ883136; int4(2)-DQ883137; int5(2)-DQ883140;
12	Albacete	Alb	nat24(1)-JF299106; nat48(1)-JF299099; nat49(1)-JF299100; nat50(1)-JF299101; nat51(1)-JF299102; nat52(2)-JF299103; nat53(1)-JF299104; nat54(1)-JF299105; nat55(1)-JF299107; int52(1)-DQ883166
13	Alcaniz	Alc	nat56(5)-JF299108, JF299109; int25(1)-DQ883159; int35(6)-DQ883165; int36(1)-DQ883176; int37(1)-DQ883185; int46(1)-DQ883163; int47(2)-DQ883164; int48(1)-DQ883180; int49(1)-DQ883181; int50(1)-DQ883182; int51(1)-DQ883191
14	Alicante	Ali	nat48(3)-JF299110,JF299113; nat49(1)-JF299114; nat57(1)-JF299111; nat58(2)-JF299112; nat59(1)-JF299115; nat60(1)-JF299116; nat61(1)-JF299117; i01(2)-DQ883129; int1(2)-DQ883129
15	Almeida	Amd	nat14(1)-JF299118; nat62(2)-JF299119; nat63(2)-JF299120; nat64(2)-JF299121; nat65(1)-JF299122; int26(4)-JF298923; int29(1)-JF298924
16	Benavente	Ben	nat66(1)-JF299123; nat67(2)-JF299124; nat68(1)-JF299125; int5(1)-DQ883141; int13(6)-DQ883148; int16(1)-DQ883142; int17(3)-DQ883143; int18(5)-DQ883144; int19(2)-DQ883145; int20(3)-DQ883147
17	Bragança	Brg	nat69(1)-JF299126; nat70(1)-JF299127; int18(1)-DQ883144; int23(3)-DQ883150; int24(4)-DQ883151,DQ883152
18	Cuenca	Cue	nat71(9)-JF299128; nat72(1)-JF299129; int62(1)-DQ883183

Continues…

Supporting Table 1a (continuation):

Locality Number	Locality	Locality Code	mtDNA haplotypes*
19	Figueira de Castelo Rodrigo	FCR	nat62(1)-JF299118; nat64(1)-JF299120; nat65(1)-JF299122; nat73(1)-JF299130; int30(3)-JF298925; int31(2)-JF298926
20	Madrid	Mad	nat74(7)-JF299131; nat75(1)-JF299132; nat76(1)-JF299133; nat77(1)-JF299134; int18(6)-DQ883146; int42(4)-DQ883167,DQ883168; int43(1)-DQ883171; int44(1)-DQ883177; int45(2)-DQ883184
21	Navarra	Nav	nat78(1)-JF299135; int1(4)-DQ883129,DQ883130; int6(2)-DQ883153; int32(3)-DQ883169; int33(11)-DQ883173; int34(2)-DQ883174
22	Salamanca	Sal	nat54(1)-JF299105; nat79(2)-JF299136; nat80(4)-JF299137; nat81(1)-JF299138; nat82(1)-JF299139; nat83(1)-JF299140; int13(2)-DQ883148; int26(1)-JF298923
23	Sendim	Sen	nat84(1)-JF299141; nat85(5)-JF299142; nat86(1)-JF299143; nat87(3)-JF299144; int24(1)-DQ883151; int27(1)-DQ883161; int28(1)-DQ883162
24	Serra da Estrela	SE	nat14(1)-JF299121; nat63(1)-JF299119; nat65(2)-JF299145; nat88(1)-JF299146; nat89(1)-JF299147; nat90(3)-JF299148; int26(2)-JF298923
25	Soria	Sor	nat24(3)-JF299149; nat91(1)-JF299150; nat92(1)-JF299151; int5(3)-DQ883140; int43(2)-DQ883171; int53(4)-DQ883170; int54(1)-DQ883172; int55(1)-DQ883175; int56(2)-DQ883188; int57(1)-DQ883189; int58(1)-DQ883192; int59(2)-DQ883193; int60(1)-DQ883194; int61(2)-DQ883178
26	Tierra de Campos	TC	nat79(4)-JF299136; nat83(1)-JF299140; nat93(2)-JF299152; nat94(1)-JF299153; nat95(1)-JF299154; int11(3)-DQ883138; int12(2)-DQ883139; int13(2)-DQ883148; int14(5)-DQ883154; int15(1)-DQ883157
27	Toledo	Tol	nat78(3)-JF299135; nat96(5)-JF299155; nat97(1)-JF299156; nat98(1)-JF299157; int1(7)-DQ883129
28	Tordesillas	Tor	nat80(2)-JF299137; nat83(4)-JF299140; nat85(1)-JF299160; nat99(1)-JF299158; nat100(2)-JF299159; int13(1)-DQ883148; int21(1)-DQ883149; int22(3)-DQ883158
29	Valencia	Val	nat101(2)-JF299161; nat102(1)-JF299162; nat103(1)-JF299163; int51(1)-DQ883179
30	Zaragoza	Zar	nat104(2)-JF299164; int1(1)-DQ883133; int7(1)-DQ883131; int8(4)-DQ883132; int9(4)-DQ883134; int10(2)-DQ883135; int35(1)-DQ883165; int36(2)-DQ883176; int37(11)-DQ883185; int38(1)-DQ883186; int39(1)-DQ883187; int40(1)-DQ883190; int41(1)-DQ883195
A and B	Majorca	Maj	**nat105(10)-KF917419**; **nat106(2)-KF917420**; **nat107(1)-KF917421**; **int63(1)-KF917422**; **int64(5)-KF917423**; **int65(1)-KF917424**; **int66(4)-KF917425**; **int67(1)-KF917426**

Continental samples used for the assignment of Majorcan specimens to their respective lineage are underlined. Haplotypes are followed by their frequencies in brackets and GenBank accession numbers. Haplotypes from sequences produced in this study are indicated in bold. Majorcan sampled populations are the localities of Llucmajor (A) and Porreres (B). *nat: *L. granatensis* native haplotypes; int: *L. granatensis timidus*-like haplotypes.

Supporting Table 1b: Populations of rabbits sampled and respective mitochondrial DNA haplotypes (absolute frequencies is depicted in parenthesis).

Locality Number	Locality	Locality Code	mtDNA haplotypes*
5	Ciudad Real	CRe	**c10(2)-KF917389**; **c11(1)-KF917390**
9	Portimão	Ptm	**a8(1)-KF917417**
16	Benavente	Bem	**c1(2)-KF917380**; **c2(1)-KF917381**; **c3(1)-KF917382**; **c4(2)-KF917383**; **c5(1)-KF917384**; **c6(1)-KF917385**; **c7(1)-KF917386**; **c8(2)-KF917387**; **c9(1)-KF917388**
24	Serra da Estrela	SE	**a7(1)-KF917418**
30	Zaragoza	Zar	**c7(2)-KF917386**; **c14(1)-KF917393**; **c15(3)-KF917394**; **c16(1)-KF917395**; **c17(3)-KF917396**; **c18(1)-KF917397**; **c19(2)-KF917398**; **c20(1)-KF917399**; **c21(1)-KF917400**
31	Huelva	Hue	**c12(1)-KF917391**
32	Lleida	Lei	**c13(1)-KF917392**
33	Arraiolos	Arr	**a1(1)-KF917409**; **a2(1)-KF917410**
34	Chaves	Chv	**a3(1)-KF917411**
35	Elvas	Elv	**a4(1)-KF917412**
36	Mirandela	Mir	**a5(1)-KF917415**
37	Mourão	Mou	**a6(1)-KF917416**
38	Galiza	Gal	**a9(1)-KF917413**; **a10(1)-KF917414**
39	France	FRA	c7(1)-AJ535805; c22(1)-AJ535811; c23(2)-AJ535810, AJ563714; c24(1)-AJ535802; c25(1)-AJ535801; c26(1)-AJ535800; c27(1)-AJ535799; c28(1)-AJ535798; c29(1)-AJ535797; c30(1)-AJ535796; c31(1)-AJ535794; c32(1)-AJ535793; c33(1)-AJ535789; c34(1)-AJ535787; c35(1)-AJ535786; c36(1)-AJ535784; c37(1)-AJ563721; c38(2)-AJ563720, AJ563716; c39(1)-AJ563719; c40(1)-AJ563718; c41(1)-AJ563715; c42(1)-AJ563713; c43(1)-AJ563712; c44(1)-AJ563711; c45(1)-AJ563710; c46(1)-AJ563709
A and B	Majorca	Maj	**c13(4)-KF917392; c32(1)-KF917408; c47(6)-KF917401; c48(3)-KF917402; c49(2)-KF917403; c50(1)-KF917404; c51(6)-KF917405; c52(1)-KF917406; c53(1)-KF917407**

Continental samples used for the assignment of Majorcan specimens to their respective lineage are underlined. Haplotypes are followed by their frequencies in parenthesis and GenBank accession numbers. Haplotypes from sequences produced in this study are indicated in bold. Majorcan sampled populations are the localities of Llucmajor (A) and Porreres (B). *c: *O. c. cuniculus* haplotyes; a: *O. c. algirus* haplotypes.

Supporting Table 2: Bayes Factor (2lnBF) estimated from the marginal likelihood calculated with the Harmonic Mean Estimator of different demographic models applied to the Majorcan hares and rabbits.

Group	Demographic model	ln Marginal Likelihood	CS	ExpoG	ExpaG	LG
Hares - *gra, nat*	Constant Size (CS)	-669.518		2.676	0.638	-
	Exponential Growth (ExpoG)	-670.855	-2.676		-2.038	-
	Expansion Growth (ExpaG)	-669.837	-0.638	2.038		-
	Logistic Growth (LG)	-	-	-	-	
Hares - *gra, tim-like*	Constant Size (CS)	-667.737		2.384	0.298	2.514
	Exponential Growth (ExpoG)	-668.928	-2.384		-2.086	0.130
	Expansion Growth (ExpaG)	-667.885	-0.298	2.086		2.216
	Logistic Growth (LG)	-668.994	-2.514	-0.130	-2.216	
Rabbits	Constant Size (CS)	-875.409		-0.444	0.684	-0.238
	Exponential Growth (ExpoG)	-875.187	0.444		1.126	0.206
	Expansion Growth (ExpaG)	-875.751	-0.684	-1.126		-0.922
	Logistic Growth (LG)	-875.290	0.238	-0.206	0.922	

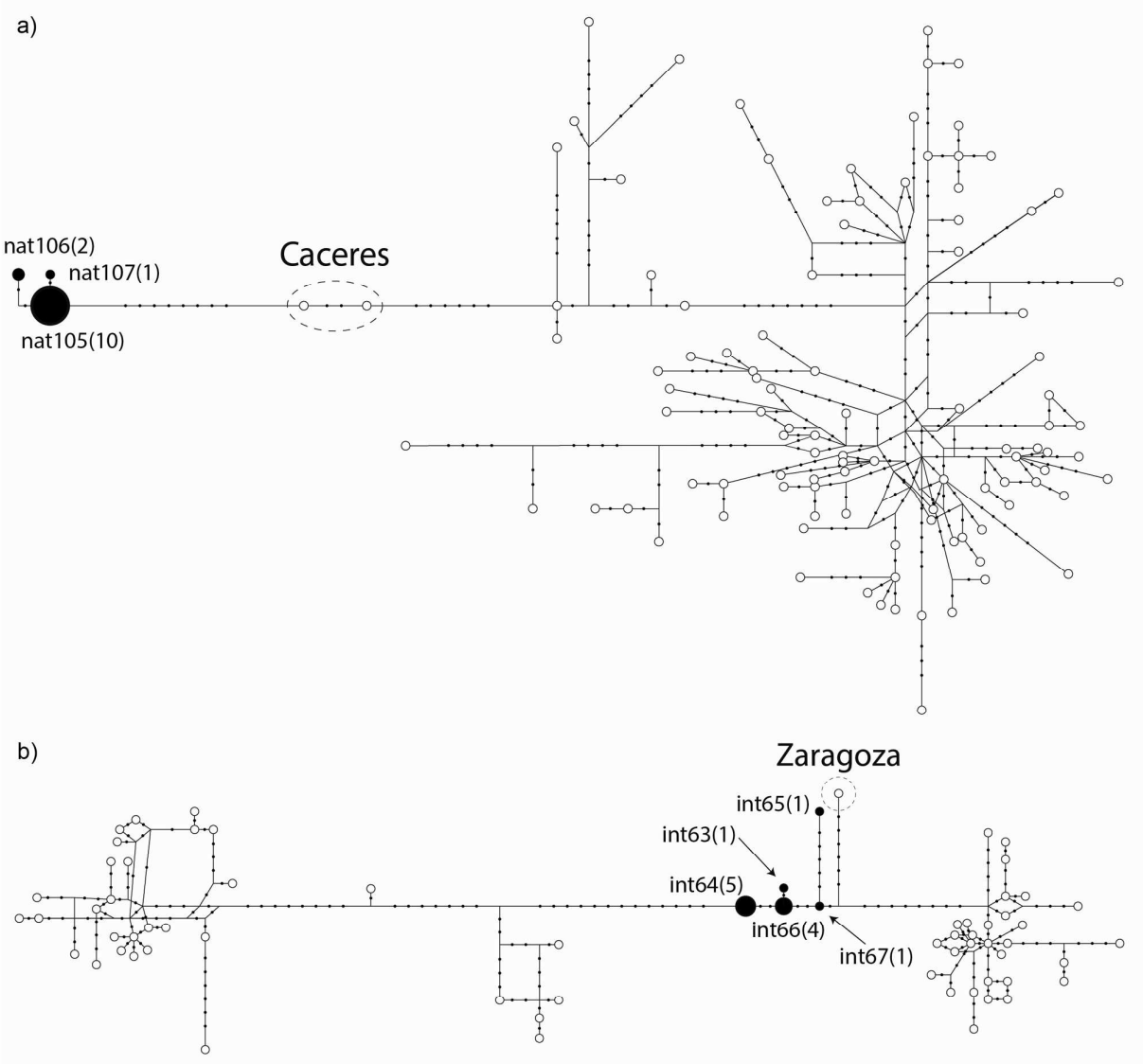
gra – *Lepus granatensis*; nat – *Lepus granatensis* with native mtDNA haplotypes; *tim-like* –*Lepus granatensis* with *timidus*-like haplotypes. Values above diagonal axis report the 2ln(Bayes factor) between the model on the left and that on the top row (below diagonal the reverse rationale applies). The analysis of logistic growth of the native *L. granatensis* haplotypes found in Majorca failed to converge and therefore the marginal likelihood and 2lnBF are not shown for this case (indicated by "-").

Supporting Table 3: Bayes Factor (2lnBF) estimated from the marginal likelihood calculated with the Path Sampling procedure of different demographic models applied to the Majorcan hares and rabbits.

Group	Demographic model	ln Marginal Likelihood	CS	ExpoG	ExpaG	LG
Hares - *gra, nat*	Constant Size (CS)	-681.016		1.804	-0.526	-
	Exponential Growth (ExpoG)	-681.918	-1.804			-
	Expansion Growth (ExpaG)	-680.753	0.526	2.330		-
	Logistic Growth (LG)	-	-	-	-	
Hares - *gra, tim-like*	Constant Size (CS)	-687.001		-0.806	-2.458	1.016
	Exponential Growth (ExpoG)	-686.598	0.806		-1.652	1.822
	Expansion Growth (ExpaG)	-685.772	2.458	1.652		3.474
	Logistic Growth (LG)	-687.509	-1.016	-1.822	-3.474	
Rabbits	Constant Size (CS)	-929.897		2.346	0.774	-2.212
	Exponential Growth (ExpoG)	-931.070	-2.346		-1.572	-4.558
	Expansion Growth (ExpaG)	-930.284	-0.774	1.572		-2.986
	Logistic Growth (LG)	-928.791	2.212	4.558	2.986	

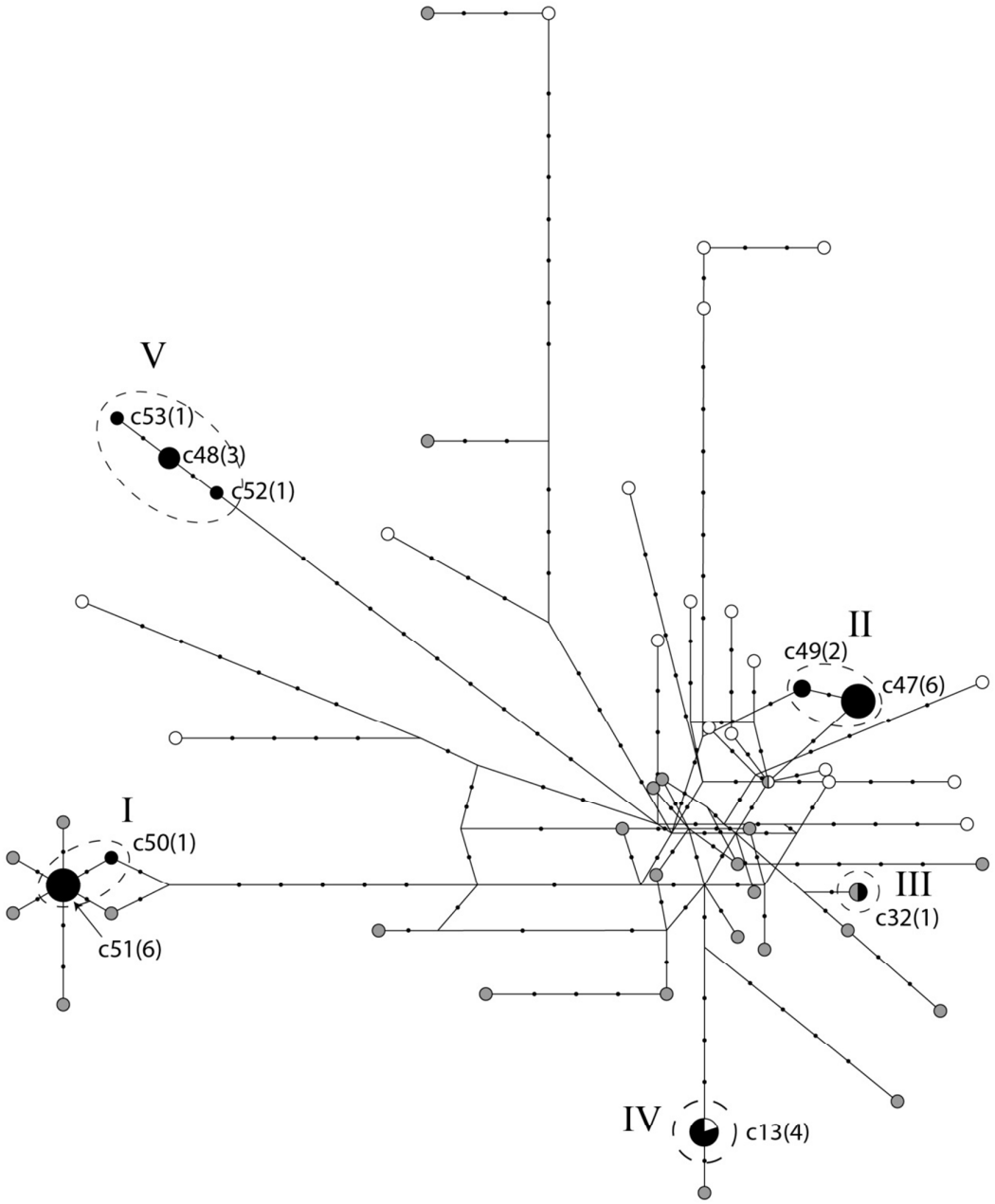
gra – *Lepus granatensis*; nat – *Lepus granatensis* with native mtDNA haplotypes; *tim-like* –*Lepus granatensis* with *timidus*-like haplotypes. Values above diagonal axis report the 2ln(Bayes factor) between the model on the left and that on the top row (below diagonal the reverse rationale applies). The analysis of logistic growth of the native *L. granatensis* haplotypes found in Majorca failed to converge and therefore the marginal likelihood and 2lnBF are not shown for this case (indicated by "-").

Supporting Figure 1:



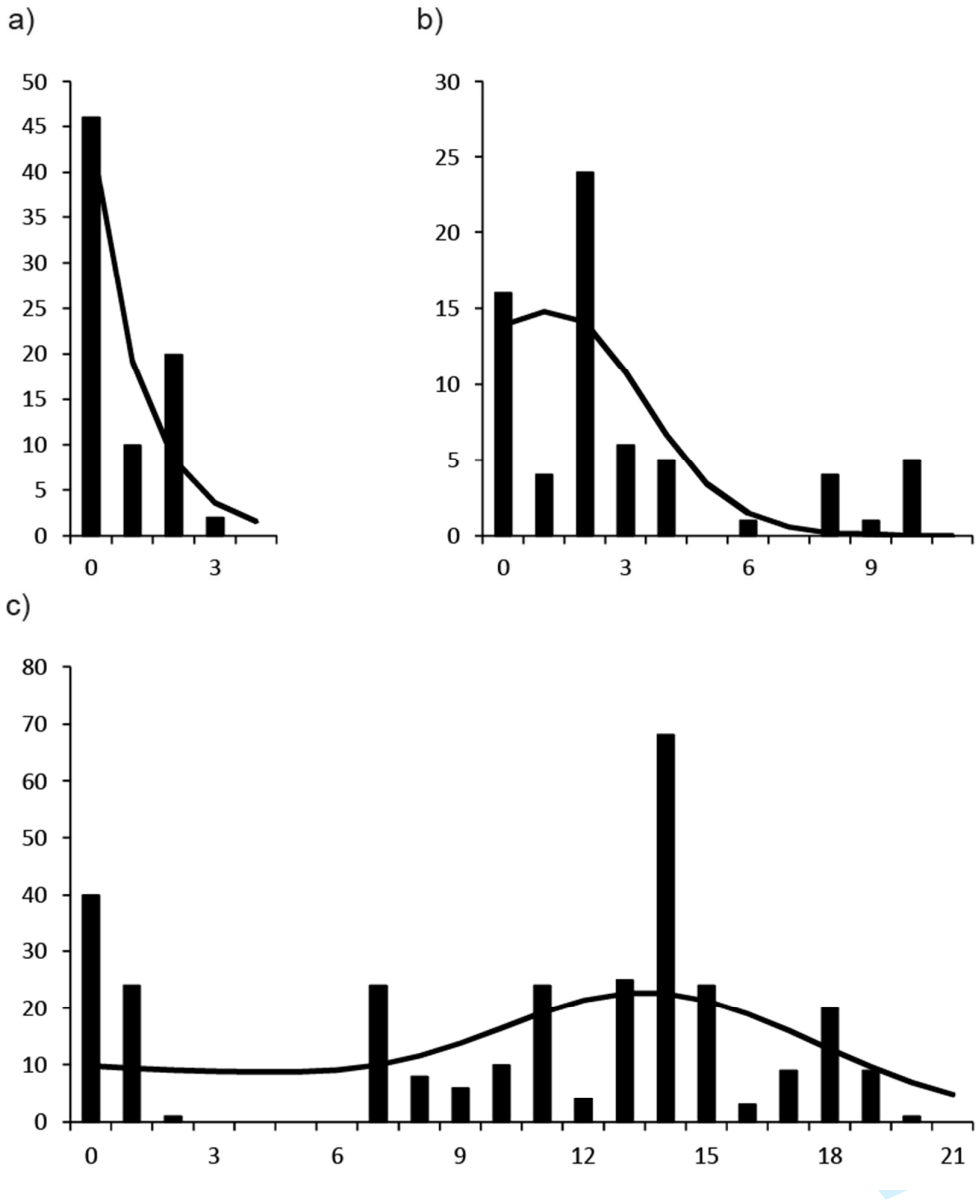
Supporting Figure 1: Median-joining network of *L. granatensis* haplotypes found in Majorca (black; haplotype sizes proportional to frequencies) and in the Iberian Peninsula (white) (a – native haplotypes; b – *timidus*-like haplotypes). Majorcan haplotype names are indicated followed by their frequency in brackets. Branches are generally proportional to the number of differences between haplotypes, and black dots on branches indicate mutational steps. Dashed circles around haplotypes indicate the closest continental haplotypes.

Supporting Figure 2:



Supporting Figure 2: Median-joining network of the rabbit lineage B haplotypes found in Majorca (black; haplotype sizes proportional to frequencies), Iberian Peninsula (white) and France (grey). Majorcan haplotype names are indicated followed by their frequency in brackets. Branches are generally proportional to the number of differences between haplotypes, and black dots on branches indicate mutational steps. Dashed circles around haplotypes indicate the Majorcan haplogroups.

Supporting Figure 3:



Supporting Figure 3: Observed (bars) and expected under a sudden expansion model (solid lines) mismatch distributions of a) Majorcan native *L. granatensis*; b) Majorcan *timidus*-like *L.granatensis*; c) Majorcan *O. c. cuniculus.* Number of pairwise differences is represented in the X-axis.